Kidney International, Vol. 66 (2004), pp. 2214–2223

The characterization of a specific Thy-1 molecular epitope expressed on rat mesangial cells

TETSUO MORIOKA, JIAN YAO, YASUHITO SUZUKI, and TAKASHI OITE

Department of Cellular Physiology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan; and Gosen Jin-Ai Clinic, Gosen, Japan

The characterization of a specific Thy-1 molecular epitope expressed on rat mesangial cells.

Background. An Experimental model of proliferative glomerulonephritis induced by an antibody against Thy-1 antigen has been established. However, the pathophysiologic role and the critical epitope of Thy-1 molecule for induction of mesangial cell dysfunction remain unknown. We have reported that monoclonal antibody 1-22-3 recognizes specific epitope which could transduce highly effective activation in mesangial cells. Identification of functional domains on cell surfaces is indispensable for understanding the molecular mechanisms of mesangial cell function. This study was undertaken to determine the functional domain containing the specific epitope recognized by monoclonal antibody 1-22-3.

Methods. A series of glutathione-S-transferase (GST)-truncated-Thy-1 proteins were generated using pGEX 4T-1 vector. COS cells were transiently transfected with plasmid vectors which could express the rat Thy-1 and mutant-Thy-1.

Results. Western blot analysis using recombinant GST-truncated-Thy-1 revealed that 1-22-3 bound to epitope at amino acids 15–23 (LRLDCRHEN). Enzyme-linked immunosorbent assay (ELISA) revealed that synthetic LRLDCRHEN peptides could inhibit the binding of 1-22-3 to rat mesangial cells and GST-Thy-1 protein. Using peptides as antigens, ELISA showed that 1-22-3 bound to the LRLDCRHEN but not to the RVNLFSDRF, which was corresponding to at amino acids 59–67 of rat Thy-1. 1-22-3 could bind the COS cells which express rat Thy-1 proteins, but could not bind rat truncated-Thy-1 which lacks residues 15–23.

Conclusion. Critical epitope detected by 1-22-3 in this study may play an important role in mesangial function and injury.

Since the concept of immunoglobulin superfamilies was proposed in 1982 [1], over 70 members have been added to the list, largely on the basis of sequence homology. There are many different functions of the family, including receptors for immunoglobulins (for example, CD16, CD89), receptors for growth factors [platelet-derived growth factor (PDGF) receptor and colony-stimulating factor-1 (CSF-1) receptor), and as adhesion molecules [carcinoembryonic antigen (CEA) and neural cell adhesion molecule (NCAM)] [2, 3]. And recently, the importance of the immunoglobulin superfamily proteins in brain development is elucidated [4, 5]. Thy-1 is a representative glycoprotein of this superfamily. Thy-1 is anchored to the cell surface via the glycosyl phosphatidylinositol (GPI) [6] and is expressed in various cell types, such as T cells, the neuron system, fibroblasts, and kidney cells [7].

Thy-1 function remains largely obscure, but it appears to be involved in cell adhesion and activation in neural and immune cells. For example, Thy-1 promotes the adhesion of thymocytes to thymic epithelia [8], the adhesion of cytotoxic T cell (CTL) clones to L cells [9], T cell activation [10], rat mast cell activation [11], and the adhesion of a Thy-1–transfected lymphoma to astrocytes [12]. Thy-1 function on rat mesangial cells is unknown. Src proteins are associated with rat mesangial Thy-1 and cross-linking of Thy-1 with antibody cause apoptosis of rat mesangial cells. From these data, we speculated that mesangial Thy-1 may act as a functional molecule. It has also been reported that some anti-Thy-1 monoclonal antibodies were capable of activating T cells, whereas others were not stimulatory [13]. It can be speculated that binding of antibodies to particular epitopes could transduce activation. Recently, we have also reported that two monoclonal antibodies, named OX-7 and 1-22-3, recognize specific epitopes which could transduce highly effective activation in mesangial cells [14].

1-22-3 binding is limited to the site of the mesangial cell surface that faces endothelial cells, and no 1-22-3 reactivity to mesangial extracellular matrix (ECM) or glomerular basement membrane (GBM) [15] was detected. By contrast, it was reported that OX-7 has reactivity toward the entire surface of mesangial cell and the mesangial cell matrix [16]. Injection of monoclonal antibody 1-22-3 into rats produced severe mesangiolysis and mesangial proliferation and much higher urinary protein excretion than seen with OX-7 [17]. On the basis of these results, we speculated that the specific Thy-1 epitope recognized

Key words: Thy-1, mesangial cell, epitope, cell adhesion molecule.

Received for publication January 27, 2004
and in revised form May 8, 2004, and June 9, 2004
Accepted for publication June 22, 2004

by 1-22-3 might play an important role in mesangial cell function and cell communication between mesangial and endothelial cells.

Identification of functional domains (epitopes) on cell surfaces is indispensable for understanding the molecular mechanisms of cell-to-cell interaction via Thy-1 molecule in physiopathologic settings. This study was undertaken to determine the functional domain containing the specific epitope recognized by monoclonal antibody 1-22-3, using recombinant fusion glutathione-S-transferase (GST)-Thy-1 proteins and synthetic peptides.

METHODS

Cells and antibodies

Rat mesangial and aortic endothelial cells were prepared as described previously [18]. Monoclonal antibodies 1-22-3 (IgG3) and OX-7 (IgG1) were produced in ascitic fluid of Balb/c mice, primed with 2,6,10,14-tetramethylpentadecane (Sigma Chemical Co., St. Louis, MO, USA). The fluid was subjected to 50% ammonium sulfate precipitation, and the immunoglobulin rich fraction was dialyzed against phosphate-buffered saline (PBS) for 2 days and stored at −70°C until use. Control monoclonal antibodies RVG1 (IgG1) and AD-4 (IgG3), confirmed to be unreactive with kidney, was also prepared from ascitic fluid as described above. The $F(ab')_2$ fragments were produced by digestion with pepsin as follows: monoclonal antibodies at 5 mg/mL in 0.1 mol/L citrate buffer, pH 3.5(OX-7) or pH 4.5 (1-22-3), were incubated at 37°C for 15 minutes (1-22-3) or 24 hours (OX-7) with pepsin (Worthington Biochemical Co., Lakewood, NJ, USA) at a final concentration of 25 μg/mL. The reaction was terminated by raising the pH to >7.0 by addition of 3.0 mol/L Tris-HCl, pH 8.6. After dialysis against PBS to remove smaller fragments, the $F(ab')_2$ fragments were separated from undigested monoclonal antibodies by superdex 200 pg gel filtration chromatography (Amersham Pharmacia, Uppsala, Sweden). Aliquots of each fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions and the appropriate fractions were collected.

Antirat CD61 (integrin-β_3 chain) monoclonal antibody was purchased from Pharmingen (BD Biosciences Pharmingen, San Diego, CA, USA). Antirat CD31 monoclonal antibody was purchased from R&D Systems (San Diego, CA, USA). Rabbit anti-FLAG polyclonal antibody was purchased from Sigma Chemical Co. Antithymocyte serum (ATS) was obtained from the rabbit, which was immunized with rat thymocytes.

Production of GST-Thy-1 fusion proteins

We constructed pGEXrThy-1 (1–111), pGEXrThy-1 (1–55), pGEXrThy-1 (51–111), pGEXrThy-1 (1–36), pGEXrThy-1 (1–22), pGEXrThy-1 (18–36), pGEXr Thy-1 (9–27), pGEXrThy-1 (13–25), pGEXrThy-1 (15–25), pGEXrThy-1 (15–27), pGEXrThy-1 (16–25), pGEXrThy-1 (15–23), pGEXrThy-1 (1–24), expression vectors for GST-Thy-1 fusion molecules, using pGEX4T-1 vector (Amersham Pharmacia). PGEXrThy-1 (a-b) represents a vector, which express GST-Thy-1 fusion protein that included residue a to b of rat Thy-1. Rat mesangial cell mRNA was isolated using the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). Reverse transcription was performed using First-Strand cDNA Synthesis Kit (Pharmacia Biotech) according to the manufacturer's information. Sets of primers carrying *Eco*RI and *Xho*I tags were designed according to each sequence to produce GST fusion proteins of those proteins. Primer sequences are listed in Table 1. Polymerase chain reaction (PCR) was carried out in an automated DNA thermal cycler using KOD DNA polymerase (Toyobo, Tokyo, Japan). The amplification protocol was 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C for 30 cycles. The PCR products and pGEX4T-1 DNA were digested with *Eco*RI and *Xho*I, ligated to each other, and then used for transformation of BL-21 cells. Inserted plasmid DNA sequences in the transformants were determined using an ABI Prism310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and appropriate cells were selected. The sequence of rat mesangial Thy-1 is 100% identical to that reported for rat Thy-1.1.

Table 1. Oligonucleotide primers used in cDNA amplification

Transcript Primer	Sequence
Sense primers	
rThy-1ms	5′-AAGAATTCCAGAGGGTGATCAGCCTG-3′
rThy-1s9	5′-AAGAATTCTGCCTGGTGAACCAGAAC-3′
rThy-1s13	5′-AAGAATTCCAGAACCTTCGCCTGGACTG-3′
rThy-1s15	5′-AAGAATTCCTTCGCCTGGACTGCC-3′
rThy-1s16	5′-AAGAATTCCGCCTGGACTGCC-3′
rThy-1s18	5′-AAGAATTCGACTGCCGTCATGAGAAT-3′
rThy-1s51	5′-AAGAATTCCCCGAGCACAACTTACCGC-3′
Antisense primers	
rThy-1mas	5′-CTCTCTCGAGACTGAAAAGGTTGAC-3′
rThy-155as	5′-CTCTCTCGAGTAAGTGTGCTCGGG-3′
rThy-136as	5′-CTCTCTCGAGGGGTCAGGCTGAACTC-3′
rThy-128as	5′-CTCTCTCGAGGGGCAAGTTGGTG-3′
rThy-125as	5′-CTCTCTCGAGTCAGGTGTTATTCTCATGAC-3′
rThy-124as	5′-CTCTCTCGAGTCAGTTATTCTCATGAC-3′
rThy-123as	5′-CTCTCTCGAGTCAATTCTCATGAC-3′
rThy-122as	5′-CTCTCTCGAGTCTCATGACGGCAGTC-3′

Purification of GST-Thy-1 peptide fusion proteins

BL21 cells harboring pGEX-Thy-1 expression constructs were induced with 1 mmol/L isopropylthio-alpha-D-galactoside for 3 hours at 37°C, harvested by centrifugation, and resuspended in PBS-0.5% Triton X-100. The suspensions were then sonicated and subjected

to centrifugation at 12,000 × *g* for 10 minutes. The supernatants were filtered with a 0.4 μm filter and then applied to a glutathione-sepharose 4B column. After extensive washing the purified GST-Thy-1 proteins were eluted with 5 mmol/L glutathione in 50 mmol/L Tris-HCl, pH 8. The purified GST fusion proteins were dialyzed against PBS and stored at −70°C until use.

SDS-PAGE and immunoblotting

BL21 cells harboring pGEX-Thy-1 expression constructs were induced with 1 mmol/L isopropylthio-alpha-D-galactoside for 3 hours at 37°C, harvested by centrifugation, and resuspended in PBS-0.5% Triton X-100. Cultured rat mesangial cells were lysed in RIPA buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS). The cell suspensions were mixed with the same volume of 2× SDS sample buffer. The cell lysate were separated in 12% SDS-polyacrylamide gels. The separated proteins were electrotransferred to 0.4 μm polyvinylidine difluoride (PVDF) membranes. The membranes were blocked with 10% milk in PBS-0.05% Tween 20 (PBS-T), pH 7.4, overnight at 4°C. After washing with PBS-T, membranes were incubated with 1 μg/mL of 1-22-3, OX-7, or mouse IgG at 4°C overnight. After extensive washing with four changes of PBS-T, filters were incubated for 1 hour at 37°C with horseradish peroxidase-conjugated sheep antimouse IgG (Bio-Rad, Richmond, CA, USA) at a 1:2000 dilution in blocking buffer. After washing, immunoreactivity was detected by the enhanced chemiluminescence (ECL) (Amersham Pharmacia) system.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates were coated with 50 μL/well of a 14 μg/mL solution of a synthetic peptide in PBS overnight at 4°C, washed with PBS-T, and then blocked by 150 μL/well of 1% bovine serum albumin (BSA) diluted in PBS-T for 1 hour at room temperature. Ten μg/mL of monoclonal antibodies 1-22-3, OX-7, and AD-4 were added to each well and plates were incubated for 1 hour at room temperature. After washing with PBS-T, peroxidase labeled antimouse IgG F(ab')$_2$-specific antibody (×3000 in blocking buffer) was added. Following a 60-minute incubation at room temperature, the plates were washed, developed with o-phenylenediamine dihydrochloride (Sigma Chemical Co.) as per manufacturer's instructions, and read with a microtiter plate reader (Bio-Rad) at 492 nm. For blocking experiments, rat mesangial cells were cultured on 96-well plates until confluent. Cells were fixed with 1% paraformaldehyde in 10 mmol/L phosphate buffer, pH 7.2, for 60 minutes and washed with phosphate buffer and blocked with 0.5% BSA in phosphate buffer at 4°C for overnight. Equal volume of 4 μg/mL of 1-22-3 or OX-7 was mixed with different concentration of synthetic peptide. And the samples were added the each well and plates were incubated for 60 minutes. After washing with phosphate buffer, peroxidase-labeled antimouse IgG F(ab')$_2$-specific antibody (×3000 in blocking buffer) was added. Following a 60-minute incubation at room temperature, the plates were washed, developed with o-phenylenediamine dihydrochloride (Sigma Chemical Co.) as per the manufacturer's instructions, and read with a microtiter plate reader (Bio-Rad) at 492 nm.

Cell surface expression of mutated rat thy-1 proteins on COS cells

For cell surface expression of Thy-1, pCDM8 mammalian expression vector was used.

We generated pCDM8rThy-1 (wild-type Thy-1), pCDM8rThy-1 (18D-E) (rat Thy-1 whose amino acid at 18 residue aspartic acid was changed to glutamic acid), pCDM8FLAGrThy-1(FLAG-tagged rat Thy-1) and pCDM8FLAGrThy-1 (15 del) (FLAG-tagged rat Thy-1 whose amino acid at 15 residue leucine was deleted), pCDM8FLAG-rThy-1 (15L-E) (FLAG-tagged rat Thy-1 whose amino acid at 15 residue leucine was replaced to glutamic acid), and pCDM8FLAGrThy-1(15L-R) (FLAG-tagged rat Thy-1 whose amino acid at 15 residue leucine was replaced to arginine). A set of primer carrying *Xba*I tags was used for generating pCDM8rThy1 construct (forward primer 5′-TAGTCTAGAATCCAGCTA TTGGCACC-3′, reverse primer 5′-TAGTCTAGATCA CAGAGAAATGAAGTC-3′). PCR was carried out in an automated DNA thermal cycler using KOD DNA polymerase (Toyobo). The amplification protocol was 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C for 30 cycles. The PCR product and pCDM8 were both digested with *Xba*I, ligated each other, and then used for transformation of MC1063/P3 cells.

Mutated Thy-1 expression vectors were produced by the method of Imai et al [19] using pCDM8rThy-1 as a original. Briefly, oligonucleotides primers were designed in inverted tail-to-tail directions to amplify the vector together with target sequences. A mutation is generated by amplification with primers that have a corresponding mutation between their 5′ ends (Table 2). After the PCR with these primers, amplified linear DNA to self-ligated, and used to transform MC1063/P3 cells. COS cells in 35 mm dishes were transfected with plasmid using polyfect transfection reagent (Qiagen, Hilden, Germany) following the manufacturer's instruction. Forty-eight hours after transfection, COS cells were processed to immunohistochemistry.

Immunohistochemistry

COS cell, rat mesangial cells, and rat aortic endothelial cells on culture glass plates were washed twice with Dulbecco's modified Eagle's medium (DMEM) and then

Table 2. Oligonucleotide primers and cDNA for construction of mutated Thy-1 expression vectors

pCDM8rThy-1 (18D-E)	
cDNA	pCDM8rThy-1
Primer 1	5′-TGCGTCATGAGAATAACACC-3′
Primer 2	5′-CTCCAGTCGAAGGTTCTGGTTC-3′
pCDM8FLAGrThy-1	
cDNA	pCDM8rThy-1
Primer 1	5′-GTCCTTGTAGTCTCCTCGGGACATCT GCAAGACTG-3′
Primer 2	5′-GACGATGACAAGCAGAGGGTGAT CAGCCTGACAGC-3′
pCDM8FLAGrThy-1 (15 del)	
cDNA	pCDM8FLAGrThy-1
Primer 1	5′-CGA CTG GAC TGC CGT CAT GAG-3′
Primer 2	5′-GTT CTG GTT CAC CAG GCA GGC-3′
pCDM8FLAGrThy-1 (15L-R)	
cDNA	pCDM8FLAGrThy-1
Primer 1	5′-CGA CTG GAC TGC CGT CAT GAG-3′
Primer 2	5′-ACG GTT CTG GTT CAC CAG GCA-3′
pCDM8FLAGrThy-1 (15L-E)	
cDNA	pCDM8FLAGrThy-1
Primer 1	5′-CGA CTG GAC TGC CGT CAT GAG-3′
Primer 2	5′-GTC GTT CTG GTT CAC CAG GCA-3′

Underlining shows the mutation or insertion sequence.

incubated with first antibody at 37°C for 30 minutes. After washing with DMEM, the specimens were incubated with fluorescein isothiocyanate (FITC)-labeled antimouse or rabbit IgG at 37°C for 30 minutes, and washed with DMEM. For double staining with 1-22-3 and OX-7, living cultured cells on culture glass plates were washed twice with DMEM and then incubated with 10 μg/mL 1-22-3 at 37°C for 30 minutes. After washing with DMEM, the specimens were incubated with FITC-labeled antimouse IgG at 37°C for 30 minutes, washed with DMEM, incubated with the second monoclonal antibody, biotinylated OX-7, for 30 minutes at 37°C, again washed with DMEM and incubated with ExtrAvidin-TRITC conjugate (60 μg/mL) (Sigma Chemical Co.) for 30 minutes at 37°C. During all the procedures using living cells, 10 mmol/L sodium azide was present in the DMEM. Finally, the treated specimens were mounted in 90% glycerol-PBS containing 0.1% p-phenylenediamine on glass slides and examined by use of confocal laser scanning microscope (MRC 1024) (Bio-Rad).

Measurements of intracellular calcium concentration

Mesangial cells were cultured to 75% confluence on a glass bottomed culture dish in DMEM with 20% fetal calf serum (FCS) for 2 to 3 days. Cells were then placed in DMEM with 0.5% FCS for 48 hours. The cells were rinsed twice with 2 mL physiologic saline solution (PSS) (135 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 10 mmol/L glucose, and 5 mmol/L Hepes, pH 7.4). The cells were then loaded with10 μmol/L fura-2/AM (Dojinn, Kumamoto, Japan) for 40 minutes at 37°C in PSS. After incubation with fura-2/AM solution, the dishes were rinsed with PSS and 1 mL of PSS was added to the each dish. One hundred μL of 1 mg/mL of $F(ab')_2$ 1-22-3 or OX-7 in PSS was added to each dish. Calcium imaging was performed on an Argus/Hisca system (Hamamatsu Photonics Ltd., Hamamatsu, Japan) with a Zeiss inverted microscope. Data acquisition and analysis was performed using the U4469–01 Argus/Hisca software. For each analysis, the dishes were placed on the microscope stage and the cells were visualized with a ×20 objective. Images were acquired at preset intervals of 3 seconds.

Cell-cell adhesion assay

Endothelial cells were plated on a 96-well culture plate and cultured until confluent. Mesangial cells were cultured in a 75 cm^2 flask and cells were labeled with ^3H-thymidine. After labeling, the cells were suspended in DMEM and incubated with 10 μg/mL of 1-22-3, OX-7, or AD-4 for 30 minutes at 4°C. After incubation the cell suspensions were added to the endothelial cells in a 96-well plate. After 20 minutes at 37°C incubation, cells were gently washed with prewarmed DMEM three times, then lysed with 1 N NaOH and the radioactivity (DPM) determined by liquid scintillation counting. Relative binding was calculated as DPM 1-22-3 or OX-7/DPM AD-4 in each experiment. For all experiments, each condition shown was performed in quadruplicate. The viability of the mesangial cells was estimated by dye exclusion assay and there was no significant difference among groups.

Statistical analysis

The results were expressed as mean ± SD, and statistical analysis was performed by Student *t* test.

RESULTS

Binding of monoclonal antibodies 1-22-3 and OX-7 to cultured mesangial cells

We examined the distribution of monoclonal antibodies 1-22-3 and OX-7 on cultured mesangial cells. The immunofluorescence showed similar diffuse, spotted binding patterns for both 1-22-3 and OX-7. Computer-aided superimposition of the two pictures enabled us to differentially localize monoclonal antibodies bound to the cultured mesangial cells. Nearly all the green-colored 1-22-3 granules on the cell surfaces changed to yellow when the images were superimposed. Furthermore, binding of OX-7 to the extracellular region was observed (data not shown).

Effect of monoclonal antibody 1-22-3 on the function of mesangial cells

One hundred μg of $F(ab')_2$ fragment of 1-22-3 or $F(ab')_2$ fragment of OX-7 was added to the mesangial

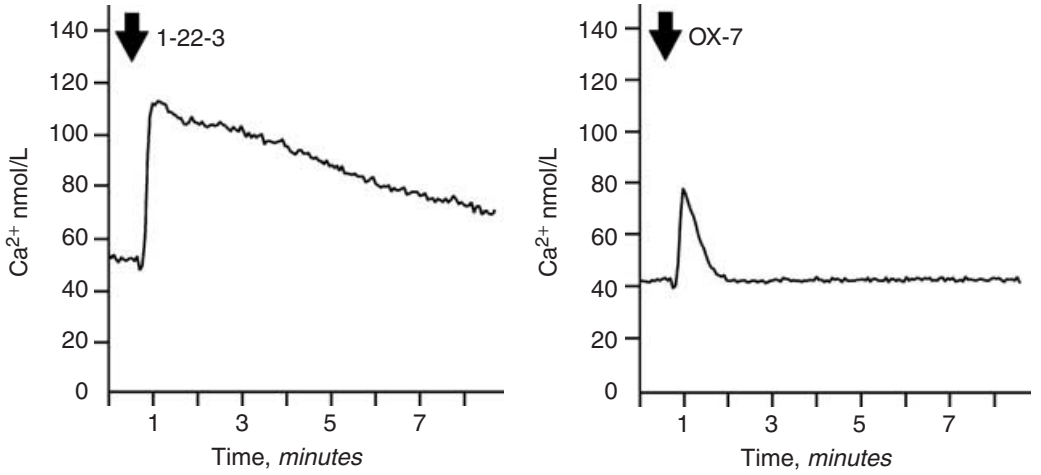

Fig. 1. Thy-1mediated increase in $[Ca^{2+}]_i$ in rat glomerular mesangial cells. Cells were prepared for fura-2 fluorescence measurements of $[Ca^{2+}]_i$ and maintained in physiologic saline solution (PSS). Calcium imaging was performed on an Argus/Hisca system. Arrow indicates the point of addition of $F(ab')_2$ antibody in PSS. $[Ca^{2+}]_i$ change in seven cells were measured in one experiment. The line exhibit the mean $[Ca^{2+}]_i$ change in the seven mesangial cells. At least four experiments were performed. This figure shows one of the representative results. Monoclonal antibody 1-22-3, basal level of 53.4 ± 4.9 to peak level of 112 ± 17.2 nmol/L, 3 minutes after addition 88.7 ± 6.6 nmol/L, 5 minutes after addition 73 ± 9.4 nmol/L ($N = 7$). Monoclonal antibody OX-7 basal level of 40.4 ± 7.9 nmol/L to peak level of 79 ± 20.3 nmol/L, 3 minutes after antibody addition 44 ± 9.3 nmol/L, 5 minutes after addition 44.3 ± 9.4 nmol/L ($N = 7$).

cell culture. 1-22-3 induced an increase in $[Ca^{2+}]i$ of the mesangial cells. The peak was at 112 ± 17.2 nmol/L and then gradually decreased for 7 to 10 minutes. OX-7 induced a transient increase in $[Ca^{2+}]_i$ of the mesangial cells, at a peak level of 79 ± 20.3 nmol/L (Fig. 1).

Rat aortic endothelial cells were stained with antirat CD61 (integrin-β_3 chain), antirat CD31 monoclonal antibody, and OX-7. Over 95% of cells were endothelial cells estimated by CD31-positive and less than 5% were OX-7-positive that means smooth muscle contamination is less than 5%. There is no positive staining for CD61 on rat endothelial cells (data not shown). Mesangial-endothelial cell adhesion was partly inhibited by the addition of 1-22-3, whereas addition of OX-7 had no effect (relative binding 0.75 ± 0.13 ($N = 4$) vs. 1.37 ± 0.10 ($N = 4$) ($P < 0.05$).

Analysis of the 1-22-3 epitope using GST-truncated-Thy-1 fusion protein

Western blot analysis showed that both 1-22-3 and OX-7 reacted with 28 kD Thy-1 molecules in RIPA extracts of mesangial cells (Fig. 2A). To determine the epitope recognized by 1-22-3, we generated GST-Thy-1 fusion proteins. 1-22-3 reacted with GST-Thy-1 (1–111), as well as with GST-Thy-1 (1–55). There was no reactivity of 1-22-3 to GST-Thy-1 (51–111). The reactivity of OX-7 to GST-Thy-1 (1–111) was much greater than with 1-22-3, whereas OX-7 showed no reactivity to both GST-Thy-1 (1–55) and GST-Thy-1 (51–111) (Fig. 2B). Then we generated the GST-truncated-Thy-1s. 1-22-3 reacted with the first 36 amino acids of the Thy-1 molecules, but with neither the first 22 amino acids nor the amino acids 18 to 36 of Thy-1 (Fig. 2C). This means that the epitope, which was recognized by1-22-3, comprises amino acids 18–22 of the Thy-1 molecule. We generated a series of GST-truncated-Thy-1 peptides (Fig. 2G). 1-22-3 recognizes the minimum sequence of 15 -23 amino acids of Thy-1 molecules (Fig. 2D to G). OX-7 had no reactivity to these GST-truncated-Thy-1s and GST-truncated-Thy-1 peptides (data not shown).

Binding of 1-22-3 to the synthetic peptide

We also examined the reactivity of 1-22-3 and OX-7 to synthetic peptides corresponding to amino acid residues 15–23 of Thy-1 by ELISA. 1-22-3 reacted with the peptides LRLDCRHEN (corresponding to amino acids 15–23 of Thy-1) but did not react with the peptide RVNLFSDRF (corresponding to amino acids 59–67 of Thy-1), whereas OX-7 had no reactivity to both peptides (Fig. 3).

Effect of the peptide LRLDCRHEN on the binding of 1-22-3 to Thy-1 and mesangial cells

To test the effect of synthetic peptide LRLDCRHEN on the binding of 1-22-3 to mesangial cells, the synthetic peptides were added to the cellular ELISA system. The peptides LRLDCRHEN could block the binding of 1-22-3 to the mesangial cells in a dose-dependent manner. However, the peptide RVNLFSDRF had no effect on the binding of 1-22-3 to the mesangial cells (Fig. 4). Both peptides could not block the binding of OX-7 to mesangial cells.

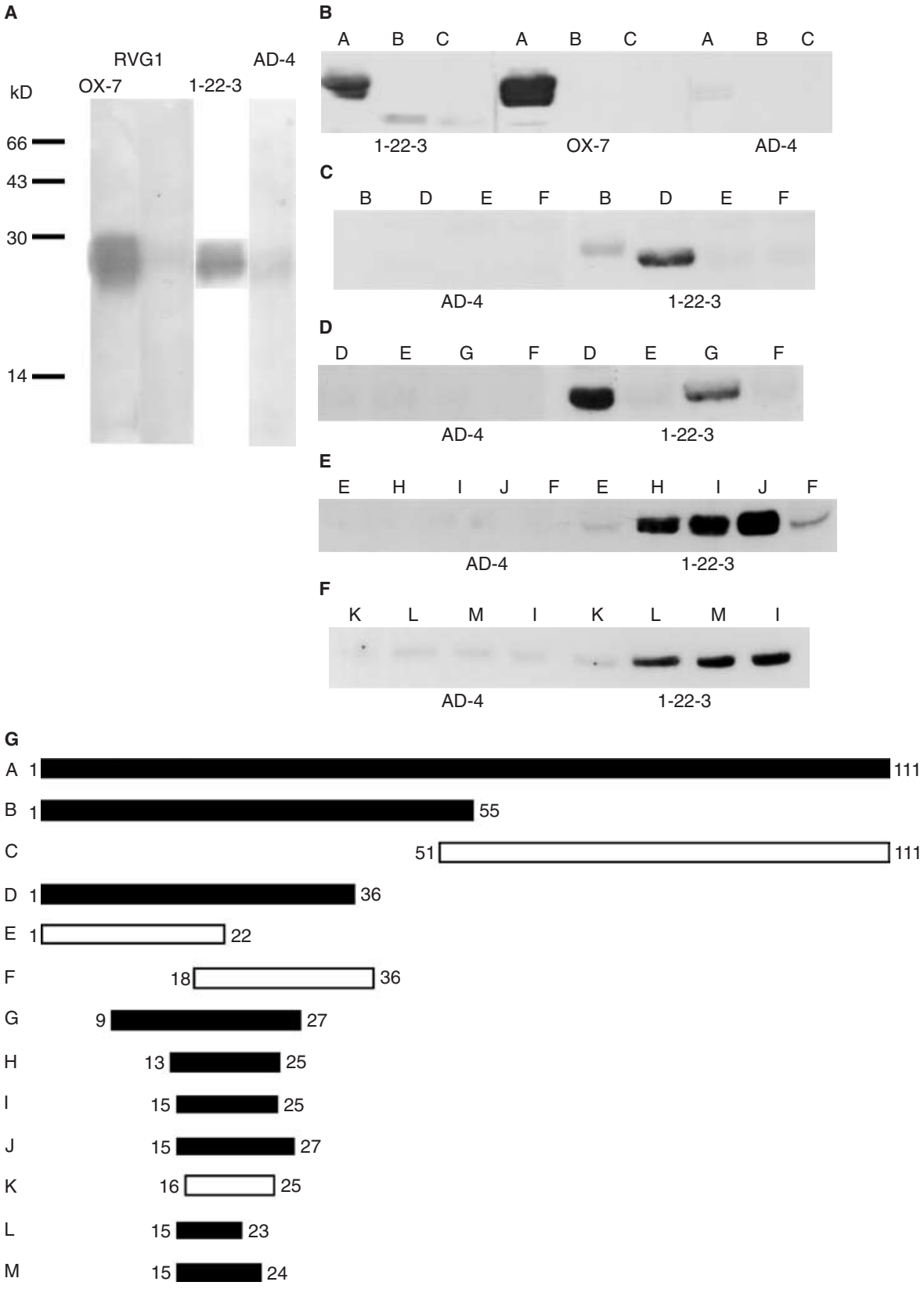

Fig. 2. Westernblot analysis of Thy-1 epitope. (*A*) Both 1-22-3 and OX-7 react with 28 kD Thy-1 molecules in RIPA extract of mesangial cells. Control antibodies RVG1 (IgG1), and AD-4 (IgG3) did not react with the RIPA extract of mesangial cells. (*B* to *F*) Total lysate from *Escherichia coli* BL21 expressing glutathione-S-transferase (GST)-truncated Thy-1 were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with 1-22-3, OX-7, and AD-4. (*G*) Schematic representation of GST-truncated Thy-1. Closed bar represents GST-truncatedThy-1 that was reacted with1-22-3.

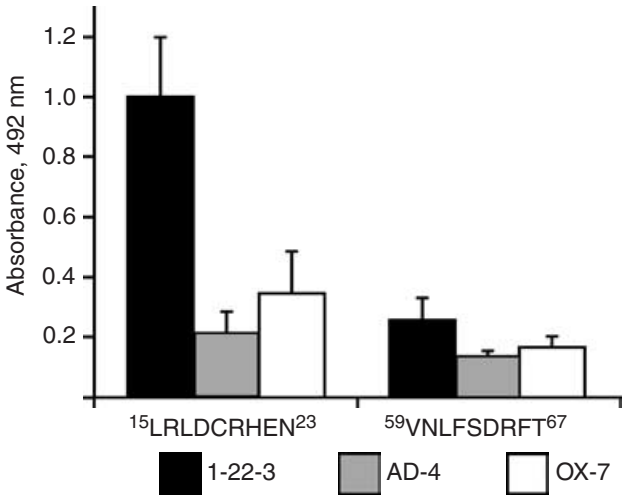

Fig. 3. Binding of 1-22-3 to the synthetic peptide. 1-22-3 binds to the synthetic peptide 15LRLDCRHEN23 but not to 59RVNLFSDRF67. OX-7 bound neither to LRLDCRHEN nor to RVNLFSDR.

Binding of 1-22-3 to mutated rat Thy-1 expressed COS cells

We made series of plasmids that could express mutated rat Thy-1 protein on COS using mammalian cell expression vector pCDM8. These proteins are anchored to the cell surface via GPI-anchor. 1-22-3 reacted the rat Thy-1 and the FLAG-tagged rat Thy-1 (Fig.5A and B). Recently it has been reported that Thy-1 binds to integrin-β_3 through Arg-Leu-Asp (RLD) sequence of Thy-1. It is reported that, when RLD sequence was changed to Arg-Leu-Glu (RLE), the binding between Thy-1 and integrin-β_3 was lost [20]. We made mutated rat Thy-1 whose RLD sequence was changed to RLE to know whether this change could influence the 1-22-3 reactivity. 1-22-3 bound to this mutated rat Thy-1. The results of GST-Thy-1 fusion protein experiments showed that the removal of residue 15 resulted the dramatic reduction of reactivity of 1-22-3 to the fusion proteins. Then we made the mutated Thy-1 whose 15 leucine was changed to arginine or glutamic acid or deleted. To make sure that the mutated rat Thy-1 is expressed on COS cells, first we made pCDM8FLAG-rThy-1. This plasmid could express FLAG-tagged rat Thy-1 when transfected to COS cells. We made then these FLAG-tagged mutated rat Thy-1 constructs. We confirmed the expression of FLAG-tagged rat Thy-1 on COS cells with anti-FLAG antibody (Fig. 5D). However, 1-22-3 did not bind these mutated rat Thy-1 (Fig. 5C). 1-22-3 and anti-FLAG antibody did not bind the COS cells that was trasfected with pCDM8 (data not shown).

DISCUSSION

In the present study, we determined the amino acid sequence of the epitope recognized by monoclonal antibody 1-22-3. A series of GST-truncated-Thy-1 fusion proteins were generated. Western blot analysis showed that 1-22-3 binds to the fusion proteins which contained residues 15–23(LRLDCRHEN) of rat Thy-1 protein [GST-rThy-1 (15–23)]. ELISA revealed that 1-22-3 binds to GST-rThy-1 (15–23), but not to fusion proteins which do not contain these residues. GST-rThy-1 (15–23) and the synthetic peptide LRLDCRHEN could, but nonrelevant peptide RVNLFSDRF could not, inhibit the binding of 1-22-3 to the mesangial cells. We also confirmed that these sequence was included in 1-22-3 epitope using mutated rat Thy-1 expressed on COS cells using mammalian cell expression vector. The results demonstrate that 1-22-3 recognized residues 15–23 (LRLDCRHEN) of rat Thy-1.

Thy-1 consists of two β sheets with antiparallel β strands. Thy-1 residues 15–23 correspond to the β strand which forms β sheets with residues 4–11 and 68–72 [21]. 1-22-3 binds to the β strands directly. Thy-1 has two allelic forms, designated Thy-1.1 and Thy-1.2 in the mouse [22, 23]. OX-7 detects an allelic variation in mouse, Thy-1.1. The difference in the primary sequence between Thy-1.1 and Thy-1.2 in the mouse is an arginine (Thy-1.1)/glycine (Thy-1.2) exchange at residue 89 [21]. Rat Thy-1, which bears the Thy-1.1 but not the Thy-1.2 determinant, also has an arginine residue at 88, which is the equivalent position to arginine 89 in mouse Thy-1.1 [21]. Position 88 on rat Thy-1.1 is included in the region that is postulated to connect β strands, and this site occurs on a sequence loop outside the membrane, this may be included in the OX-7 epitope. Antibody cross-linking of GPI-anchored proteins, including Thy-1, leads to protein phosphorylation, intracellular calcium mobilization [24]. The exact mechanism of signal transduction in GPI-anchor protein is not yet known. The current understanding of the GPI-anchored protein signaling process is that cross-linking leads to coalescence of microdomains, which generates threshold levels of activation signals through clustered microdomainassociated Src family protein tyrosine kinases (PTKs) [25, 26].

We speculate that the difference of the binding site of antibody may affect the coalescence rate and the efficiency of signal transduction, although we do not have direct evidence.

The cleavage of the GPI-anchor from Thy-1 resulted in a loss of antigenic activity, detected by OX-7 [27, 28]. This suggests that the epitope, which was recognized by OX-7, may be conformational dependent. In our study, OX-7 bound to GST-rThy-1 (1–111), but bound neither to GST-rThy-1 (1–55) nor to GST-rThy-1 (52–111). The epitope that was recognized by OX-7 may require both parts and residues 52–111 may be not sufficient to form the structure recognized by OX-7. OX-7 bound to mutated Thy-1, whose GPI-anchor was replaced by the three amino acids Gly Gly Ser [29], and also bound to

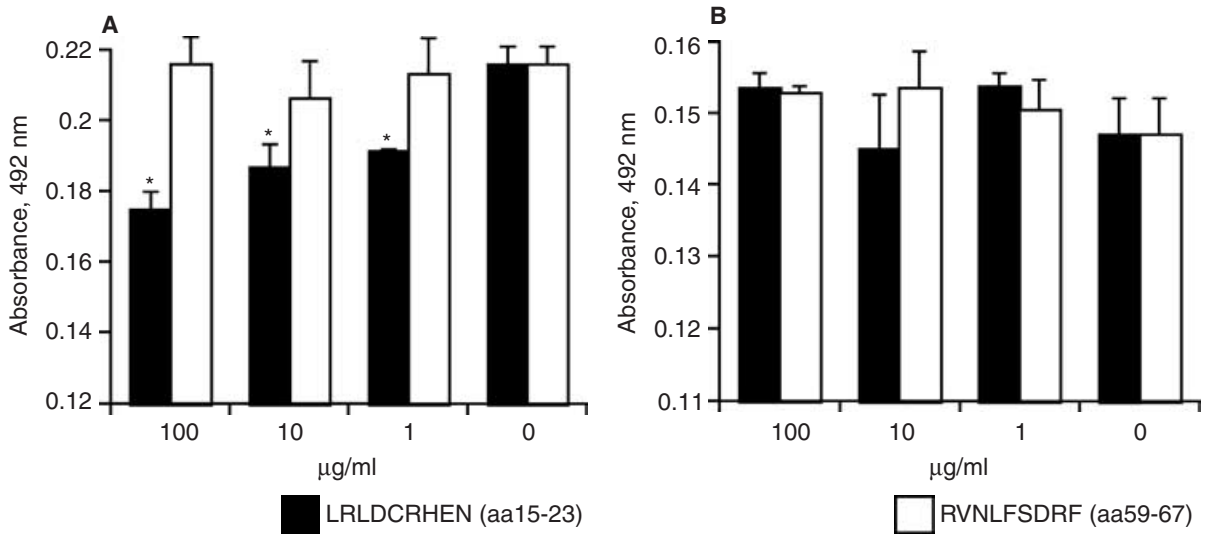

Fig. 4. LRLDCHREN blocks the binding of 1-22-3 to rat mesangial cells. 15LRLDCHREN23 peptide (*A*) inhibits the binding of 1-22-3 to rat mesangial cells in a dose dependent manner, but 59RVNLFSDRF67 peptide (*B*) had no effect on the binding. Both peptides had no effect on the binding of OX-7 to rat mesangial cells. $*P < 0.05$ compared to control ($N = 4$).

Thy-1-immunoglobulin fusion protein [30]. The extra amino acids or protein presumably stabilize the Thy-1 in a similar manner to the GPI anchor [29]. In our experiment, GST may have stabilized the Thy-1 in a similar manner to the GPI anchor.

The biologic significance of the Thy-1 molecule on rat glomerular mesangial cells remains unknown, although a rat model of anti-Thy-1 antibody induced glomerulonephritis with mesangial cell injury has been established and utilized to clarify the mechanisms responsible for mesangial cell injury and inflammation [31–33]. We have reported that administration of a single injection of the monoclonal antibody against Thy-1 antigen used in the present study, 1-22-3, induced massive proteinuria in rats and that the nephritogenicity of this monoclonal antiboy, assessed by estimating the severity of proteinuria and glomerular inflammation induced after injection, appeared to be higher than that caused by the well-known anti-Thy-1.1 monoclonal antibody OX-7 [17, 34]. The different nephritogenicities of these two monoclonal antibodies may be explained by differences in their complement-fixing activities, which are dependent on the IgG subclass: 1-22-3 belongs to the IgG3 subclass and OX-7 is an IgG1.In fact, an in vitro complement-dependent cytotoxicity assay showed that 1-22-3 had higher cytotoxic activity against cultured rat mesangial cells than the OX-7 (preliminary experiment). However, another possibility is that the specific epitope recognized by 1-22-3 is a critical epitope for mesangial cell function.

We have shown, using whole antibody, that 1-22-3 and OX-7 induced the calcium increase in mesangial cells. The peak level of calcium concentration and the sustained duration of calcium increase was much greater than 1-22-3. The shape of curves of the calcium response was

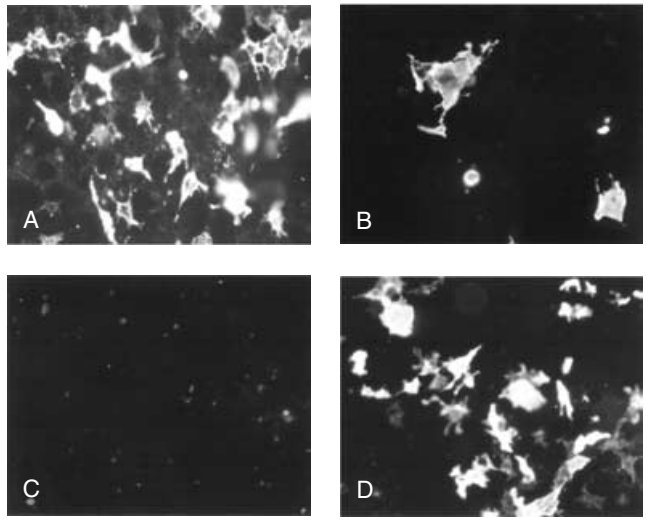

Fig. 5. COS cells were transfected with mutated rat Thy-1 expressing plasmids. (*A*) RatThy-1. (*B*) FLAG tagged rat Thy-1. (*C*) FLAG-tagged rat Thy-1 (15L-R). (*D*) FLAG-tagged rat Thy-1 (15L-R). COS cells were stained with 1-22-3 [10 μg/mL in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% azide (DME azide)] (A to C) or anti-FLAG polyclonal antibody (50 μg/mL in DME azide) (D),washed with DME azide three times and then stained with fluorescein isothiocyanate (FITC)-labeled antimouse IgG antibody for 1-22-3 or FITC-labeled antirabbit IgG antibody for anti-FLAG antibody (original magnification ×400).

not exactly the same as that in previous experiment. This difference may explain that the different preparation of antibodies and the method we used for measuring the calcium might influence the results. We could not completely exclude the effect of complement using whole antibodies. Then, in the present paper using $F(ab')_2$ antibodies, we showed that both 1-22-3 and OX-7 could increase the intracellular free calcium concentration. 1-22-3 induced

a sustained increase in calcium concentration, while that induced by OX-7 was transient, lasting 1 minute, then decreased to basal level. We have also found that, in serum-free conditions, incubation of mesangial cells with 1-22-3 resulted in a significantly increased inositol-1,4,5 triphosphate (IP_3) level and phosphorylation of src kinases, than seen with OX-7 [14, 35]. These results suggested that the epitope recognized by 1-22-3 effectively transduce signals in mesangial cells, independent of complement.

Previously we have reported that the epitope recognized by 1-22-3 was concentrated at the site on the cell surface that faced the neighboring endothelial cells, when cocultured with vascular endothelial cells [36]. In addition, mesangial cell proliferation regressed as a result of intercellular contact with endothelial cells [37], although the molecular mechanism of cell contact-dependent growth inhibition remains unclear. In the present study, we showed that 1-22-3 could partially inhibit the binding of mesangial cells to endothelial cells. These data suggest that the epitope may be important for mesangial-endothelial cell interactions.

The ligand of Thy-1 has not been totally clarified. Homophilic interaction of Thy-1 could be one of the candidates [38]. Recently, Leyton et al [20] reported that Thy-1 binds to integrin-β_3 and triggers tyrosine phosphorylation of focal adhesion proteins in astrocytes. Thy-1 has an RGD-like sequence RLD. Through the RLD sequence, Thy-1 binds to integrin-β_3 [20]. This RLD sequence was contained in the epitope that was recognized by 1-22-3. However, rat glomerular endothelial cells and cultured rat aortic endothelial cells did not express integrin-β_3 in our experimental conditions. And Thy-1 was not expressed on cultured rat aortic cells in our experimental condition. Further studies are needed to clarify the ligand molecule of Thy-1 expressed on the rat mesangial cells.

Taken together, we speculate that disturbances of communication between mesangial and endothelial cells, caused by impairment of vascular regeneration [39] and matrix remodeling, may induce pathological expansion of the mesangium, eventually leading to glomerulosclerosis.

Analysis of the molecular mechanisms involved in endothelial and mesangial cell contact, including Thy-1 molecules, may help us to understand the mechanisms and develop new strategies for inhibition of progressive glomerulosclerosis.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for scientific research (C:10670988, 12671032; B:15390266) from the Japanese Ministry of Education, Science, Sports and Culture. The authors wish to thank Dr. S. Batsford, Institute of Medical Microbiology and Hygiene, Freiburg University, Germany, for critical review and advice on the manuscript and Dr. F. Shimizu, Institute of Nephrology, Niigata University, for his generous support. We thank for Mr. Shigeru Hirayama, Asahimachi Laboratory of Radioisotope Research, Niigata University, for his help with radioisotope experiments and Miss Keiko Kamata for her technical assistance.

Reprint requests to Tetsuo Morioka, M.D., Niigata University Graduate School of Medical and Dental Science, Department of Cellular Physiology, Institute of Nephrology, 951–8510 Asahimachi-dori 1–757, Niigata, Japan.
E-mail: morioka@med.niigata-u.ac.jp

REFERENCES

1. Williams AF: Surface molecules and cell interactions. *J Theor Biol* 98:221–234, 1982
2. Williams AF: A year in the life of immunoglobulin superfamily. *Immunol Today* 8:298–303, 1987
3. Holness CL, Simmons DL: Structural motifs for recognition and adhesion in members of the immunoglobulin superfamily. *J Cell Sci* 107:2065–2070, 1994
4. Kamiguchi H, Lemmon V: IgCAMs: Bidirectional signals underlying neurite growth. *Curr Opin Cell Biol* 12:598–605, 2000
5. Rougon G, Hobert O: New insights into the diversity and functin of neuronal immunoglobulin superfamily molecules. *Ann Rev Neurosci* 26:207–238, 2003
6. Homans SW, Ferguson MA, Dwek RA, *et al*: Complete structure of the glycosyl phosphatidylinositol membrane anchor of rat brain Thy-1 glycoprotein. *Nature* 333:269–272, 1988
7. Barclay AN, Brown MH, Law SKA, *et al*: *Leukocyte Antigen Factsbook*, 2nd ed., London, Academic Press, 1997
8. He HT, Naquet P, Caillol D, *et al*: Thy-1 supports adhesion of mouse thymocytes to thymic epithelial cells through a Ca^{2+} independent mechanism. *J Exp Med* 173:515–518, 1991
9. Johnson R, Lancki DW, Fitch FW: Accessory molecules involved in antigen-mediated cytolysis and lymphokine production by cytotoxic T lymphocyte subsets. I. Identification of functions for the T cell surface molecules Ly-6C and Thy-1. *J Immunol* 151:2986–2999, 1993
10. Gunter KC, Malek TR, Shevach EM: T cell-activating properties of an anti-Thy-1 monoclonal antibody. Possible analogy to OKT3/Leu-4. *J Exp Med* 159:716–730, 1984
11. Draberova L: The involvement of Thy-1 antigen in the activation of rat mast cells. *Eur J Immunol* 19:1715–1720, 1989
12. Dreyer EB, Leifer D, Heng JE, *et al*: An astrocytic binding site for neuronal Thy-1 and its effect on neurite outgrowth. *Proc Natl Acad Sci USA* 92:11195–11199, 1995
13. Pont S, Regnier-Vigouroux A, Naquet P, *et al*: Analysis of the Thy-1 pathway of T cell hybridoma activation using 17 rat monoclonal antibodies reactive with distinct Thy-1 epitopes. *Eur J Immunol* 15:1222–1228, 1985
14. Narisawa-Saito M, Kimura S, Fujiwara N, *et al*: Thy-1-mediated phosphatidylinositol turnover in cultured rat glomerular mesangial cell. *J Cell Physiol* 168:705–710, 1996
15. Kawachi H, Orikasa M, Matsui K, *et al*: Epitope-specific induction of mesangial lesions with proteinuria by a MoAb against mesangial cell surface antigen. *Clin Exp Immunol* 88:399–404, 1992
16. Paul LC, Rennke HG, Milford EL, *et al*: Thy-1.1 in glomeruli of rat kidneys. *Kidney Int* 25:771–777, 1984
17. Nakayama H, Oite T, Kawachi H, *et al*: Comparative nephritogenicity of two monoclonal antibodies that recognize different epitopes of rat Thy-1.1 molecule. *Nephron* 78:453–463, 1998
18. Oite T, Suzuki Y, Morioka T, *et al*: Efficient isolation of rat aortic endothelial cells by elimination of contaminating cells with a monoclonal antibody. *Microvasc Res* 50:113–118, 1995
19. Imai Y, Matsushima Y, Sugimura T, *et al*: A simple and rapid method for generating a deletion by PCR. *Nucleic Acids Res* 19:2785, 1991
20. Leyton L, Schneider P, Labra CV, *et al*: Thy-1 binds to integrin beta(3) on astrocytes and triggers formation of focal contact sites. *Curr Biol* 11:1028–1038, 2001
21. Williams AF, Gagnon J: Neuronal cell Thy-1 glycoprotein: Homology with immunoglobulin. *Science* 216:696–703, 1982
22. Reif AE, Allen JM: The AKR thymic antigen and its distribution in leukemias and nervous tissues. *J Exp Med* 120:413–433, 1964
23. Letarte M, Meghji G: Purification and characterization of mouse brain Thy-1 glycoprotein antigens. *J Immunol* 121:1718–1725, 1978

24. Sharom FJ, Lehto MT: Glycosylphosphatidylinositol-anchored proteins: Structure, function, and cleavage by phosphatidylinositol-specific phospholipase C. *Biochem Cell Biol* 80:535–549, 2002
25. Simmons K, Ikonen E: Functional rafts in cell membranes. *Nature* 387:569–572, 1997
26. Harder T, Scheiffele P, Verkade P, *et al*: Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J Cell Biol* 141:929–942, 1998
27. Barboni E, Rivero BP, George AJ, *et al*: The glycophosphatidylinositol anchor affects the conformation of Thy-1 protein. *J Cell Sci* 108:487–497, 1995
28. Kukulansky T, Abramovitch S, Hollander N: Cleavage of the glycosylphosphatidylinositol anchor affects the reactivity of thy-1 with antibodies. *J Immunol* 162:5993–5997, 1999
29. Devasahayam M, Catalino PD, Rudd PM, *et al*: The glycan processing and site occupancy of recombinant Thy-1 is markedly affected by the presence of a glycosylphosphatidylinositol anchor. *Glycobiology* 9:1381–1387, 1999
30. Lee WS, Jain MK, Arkonac BM, *et al*: Thy-1, a novel marker for angiogenesis upregulated by inflammatory cytokines. *Circ Res* 82:845–851, 1998
31. Border WA, Okuda S, Languino LR, *et al*: Transforming growth factor-beta regulates production of proteoglycans by mesangial cells. *Kidney Int* 37:689–695, 1990
32. Johnson RJ, Raines EW, Floege J, *et al*: Inhibition of mesangial cell proliferation and matrix expansion in glomerulonephritis in the rat by antibody to platelet-derived growth factor. *J Exp Med* 175:1413–1416, 1992
33. Shimizu A, Kitamura H, Masuda Y, *et al*: Apoptosis in the repair process of experimental proliferative glomerulonephritis. *Kidney Int* 47:114–121, 1995
34. Kawachi H, Oite T, Shimizu F: Quantitative study of mesangial injury with proteinuria induced by monoclonal antibody 1-22-3. *Clin Exp Immunol* 92:342–346, 1993
35. Narisawa-Saito M, Yamanashi Y, Morioka T, *et al*: Thy-1 molecule associates with protein tyrosine kinase(s) in rat mesangial cells. *Clin Exp Immunol* 106:86–90, 1996
36. Oite T, Saito M, Suzuki Y, *et al*: A specific Thy-1 molecular epitope expressed on rat mesangial cells. *Exp Nephrol* 4:350–360, 1996
37. Saeki T, Morioka T, Arakawa M, *et al*: Modulation of mesangial cell proliferation by endothelial cells in coculture. *Am J Pathol* 139:949–957, 1991
38. Millrain MM, Hederer R, Griffiths S, *et al*: Novel cell junctions induced by activating Thy-1-specific antibodies. *Int Immunol* 4:1137–1143, 1992
39. Wada Y, Morioka T, Oyanagi-Tanaka Y, *et al*: Impairment of vascular regeneration precedes progressive glomerulosclerosis in anti-Thy 1 glomerulonephritis. *Kidney Int* 61:432–443, 2002